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Azadirachtin effects on reproductive traits and progeny survival in *Drosophila melanogaster* (Diptera)

Running title: Azadirachtin reproductive toxicity

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ABSTRACT

BACKGROUND - Azadirachtin is a prominent natural pesticide and represents an alternative to conventional insecticides. It has been successfully used against insect pests but has also various effects on non-target organisms, that still are poorly known. Here, we investigated lethal and sublethal effects of Azadirachtin on treated adults of *Drosophila melanogaster* (Meigen) and examined determined and several parameters of reproduction (fertility, spermatogenesis, and oogenesis) ..

RESULTS – Azadirachtin, applied topically on newly emerged adults, increased mortality with a positive dose-dependent relationship. The LD_{50} (0.63 μg) was determined 24 hours after treatment using a non-linear regression. Azadirachtin induced a significant reduction in reproductive potential in treated flies, particulary when females were treated. In addition, the number of cysts and the apical nuclei positions within the cysts decreased by 29.7% and 20% respectively in males. In females, the number of oocytes per ovary and the volume of basal oocytes also decreased by 16.1% and 32.4% respectively.

CONCLUSION - Azadirachtin causes significant toxic effects in both sexes and decreases the fecundity in non-targeted species. Females are more sensitive to Azadirachtin.

Key words: natural pesticide, Azadirachtin, toxicity, spermatogenesis, oogenesis, *Drosophila*

I. INTRODUCTION

Crop losses caused by pests may represent the equivalent of the food needed to feed over 1 billion people.[1] The use of pesticides is therefore required to protect agrosystems. However, their side effects induce important health[2,3] and environmental problems.[4,5] Consequently, natural compounds with pesticidal properties (biopesticides) have been investigated for decades and offer a more sustainable solution to pest control than synthetic or conventional products.[8]

Azadirachtin, a tetranortriterpenoid derived from the seeds of the Indian neem tree (*Azadirachta indica* A. Juss, Meliaceae), is one of the main commercialized biopesticides and remains the most successful botanical pesticide in agricultural use worldwide.[9,10] It possesses a strong toxicity against insect pests of different orders.[9,11-14] Besides its insecticidal action, Azadirachtin is also used in traditional medicine, in Asia and in Africa as an antidiabetic, immunostimulant, antimicrobial, antiviral, contraceptive and anticancer remedy.[15-17]

Azadirachtin is an insecticide with rapid biodegradability and without resistance problems due to its chemical complexity.[9] This pesticide is also reported to be relatively safer than most conventional insecticides.[18,19] It is non-toxic to humans and warm-blooded vertebrates[20] and without genotoxicity for mammals.[21,22] However, several studies have questioned the safety of neem products on non-target species, but the results remain contradictory.[23-28] For example, behavior and morphological alterations have been highlighted in bees,[28] while Bernades *et al*.[26] did not note any effect on worker bee mortality, flight, or respiration rate; furthermore, in aquatic organisms, Azadirachtin is used as a potential immunostimulant and a promising agent to control fish parasites.[27] Because Azadirachtin acts as an insect growth disruptor[29] negative side effects on beneficial arthropods are expected.

Azadirachtin interferes with the endocrine system and interacts with juvenile hormone and 20-hydroxyecdysone. It induces growth and moulting abnormalities and affects ovarian

development, oocyte structure, fecundity, oviposition and egg viability.[9, 12,30-32] Azadirachtin also shows moderate to strong cytotoxicity, neurotoxicity and antimitotic effects.[9,33-36] It acts on the expression of genes related to development in *Drosophila melanogaster*.[18] In addition, varied effects have been noted according to the tested Azadirachtin formulation.[12,37] Nevertheless, the mechanism of action of this pesticide remains elusive.[18,19]

Direct effects of Azadirachtin on various developmental processes, related to the signaling of 20-hydroxyecdysone, have been previously reported.[9] However, the mechanisms of action of Azadirachtin on reproduction are still poorly known. Azadirachtin has been shown to influence oviposition behavior in several insects,[11,38] but its precise effects on reproductive parameters would benefit from being more deeply investigated. Therefore, the aim of this study was double: on the one hand, it quantified the side effects of Azadirachtin on the non-target insect *D. melanogaster* using doses allowing 25 and 50% of survivors, *i.e.* mimicking possible exposure to the insecticide residues; on the other hand, it filled some gaps in evaluating Azadirachtin reprotoxic effects in this biological model species. Effects were assessed (1) following treatment on newly emerged adults, and (2) on various reproductive traits of adult males and females. Previous studies have already shown that Azadirachtin impacts the reproduction of *D. melanogaster* treated at the pupae[39] and larvae stages.[38] Thus, we considered its impact on the survival of progeny at various development stages and on fertility in both sexes. We quantified the number of descendants produced by surviving adults (numbers of eggs, larvae, pupae and adults obtained at the F_1 generation) and also the fecundity of males and females (number of cysts and potential anomalies of spermatocyte nucleus positions; number of oocytes and size of basal oocyte). A toxicity study was first carried out to determine lethal doses for *D. melanogaster* adults in our experimental conditions.

2. MATERIAL AND METHODS

2.1 Insect rearing

Drosophila melanogaster (Canton-S) was reared on standard cornmeal medium at 25 ± 2°C, 70% relative humidity under a 12-hour light/dark photoperiod. Flies were transferred every three days to avoid larval competition and to regularly provide abundant progeny for testing.

2.2 Insecticide and toxicity tests

The Neem Azal (1% Azadirachtin; TrifolioM GmbH; Lahnau, Germany) commercial formulation, was dissolved in acetone and topically applied on adults (sexes combined after screening and showing any difference between male and female) less than 6 hours after ecdysis (1 µl per insect according to Di Prisco *et al*.).[40] Control insects were treated with solvent alone. Five doses (0.1, 0.2, 0.4, 0.6 and 1.2 µg of Neem Azal acetone solution) were tested,[39] considering 3 replicates per dose, each consisting of 300 insects. Adult mortality percentages were calculated between 24 and 96 hours after treatment and corrected in accordance with Abbott.[41] A non-linear regression was used to determine the lethal doses (LD) LD_{25} and $LD_{50,}$ corresponding to 25% and 50% of adult mortality respectively, with their corresponding 95% fiducial limits (95% FL) and Hill slope.

2.3 Progeny output

Newly moulted (< 6 hours) *D. melanogaster* adults were treated topically with Azadirachtin at the LD_{25} and LD_{50}. Subsequently, one male and one female from the control and treated series were placed in a Petri dish (90 x 14.2 mm) containing standard cornmeal medium to obtain their progeny. Eight repetitions of different types of couples were realized (control male + control female; control male + female LD_{25}; male LD_{25} + control female; male $LD_{25,}$ + female $LD_{25;}$ control male + female LD_{50}; male LD_{50} + control female; male LD_{50} + female LD_{50}). After 48 hours, the adults were removed and generation 1 (F_1) was followed daily until all

adults had emerged. The numbers of eggs, larvae, pupae and adults that emerged from each series of experiments were counted.

2.4 Cyst parameters in males

Developmental factors leading to male infertility were investigated by analyzing post-meiotic abnormalities, *i.e*. cyst numbers and spermatocyte nucleus localization, both parameters influencing the number of sperm produced.[42] Newly moulted male adults (< 6 hours) were treated topically with Azadirachtin at the LD_{50} (0.63 µg). Individuals from the control and treated series were placed in vials containing standard cornmeal medium. After 48 hours, testes were dissected in a phosphate-buffered saline (PBS) solution with 4', 6-diamidino-2-phenylindole (DAPI) allowing nuclei staining.[43] Cysts were gently spread on the blade and covered with a cover slip. Cysts were then observed under a fluorescence microscope and the number of cysts per male and the position of spermatocyte nuclei within the cyst were counted. Thirty repetitions per condition were performed.

2.5 Ovarian parameters in females

To detect a possible impact of Azadirachtin on female fecundity, classical ovarian parameters were investigated (number of oocytes and size of basal oocyte). Newly emerged female adults (< 6 hours) were treated topically with the Neem Azal at the LD_{50} (0.63 µg). Individuals from the control and treated series (LD_{50}: 0.63 µg) were placed in vials containing standard cornmeal medium. After 48 hours, ovaries were dissected out. After removal of the circumovarian fat body, the numbers of oocytes were scored together with the volume of the basal oocyte.[44]

2.6 Statistical analysis

The mean ± standard deviations (SD) were calculated for each experimental group. Data from the toxicity assay were analyzed using non-linear sigmoid curve fitting. The goodness of fit to the curve model was evaluated on the basis of R^2 values. The homogeneity of variances was

checked using Bartlett's and Shapiro Wilk Tests. Analyses of variance (one-way and two-way) were performed and followed by Tukey's HSD test for multiple comparisons when significant. Repeated-measure Anova was also used for progeny output between stages for the same couple. Numbers of cysts and ovarian parameters were compared between the treated and control groups using Student's t test. All calculations were performed using GraphPad Prism (v6.01 for Windows).

3. RESULTS

3.1 Insecticidal activity

Azadirachtin, applied topically on newly emerged adults of *D. melanogaster*, induces an insect mortality, 24 hours after treatment. Corrected mortality percentages were 13.92 ± 1.43 % for the lowest dose (0.1 µg) and 71.80 ± 3.11 % for the highest dose (1.2 µg). The mortality percentage recorded in untreated animals was 5.48 ± 0.53 % (control mortality, Fig.1). Statistical analysis revealed a significant dose effect ($F_{4, 10} = 62.43$; $p < 0.0001$) and Tukey's HSD test showed a significant increase in mortality with increasing doses (Fig. 1). The lethal doses (LD) recorded with 95% fiducial limits (95% FL) were $LD_{25} = 0.23$ (0.13-0.38), $LD_{50} = 0.63$ (0.44-0.91) and $LD_{90} = 4.85$ (1.47-15.98) µg (Table 1). The non-linear regression fitted on these results indicated a Hill slope of 1.08 (0.54-1.62). No significant effect of the time elapsed after treatment (24 to 96 hours) was observed ($F_{5, 60} = 1.05$; $p = 0.39$; Table 2).

3.2 Progeny output

To evaluate the progeny from surviving treated adults, we monitored the different developmental stages (eggs, larvae, pupae and adults) in the progeny of couples formed with control animals (female, F_C; male, M_C) and/or animals treated with the LD_{50} (female, F_T, male M_T). Statistical analysis showed a significant decrease in the numbers of eggs, larvae, pupae and adults in the F_1 generation for all treated series compared to couples from control

individuals (Repeated-measure ANOVA: treatment effect: $F_{6, 42} = 241.90$, $p < 0.0001$; stage effect: $F_{3, 21} = 15.94$, $p < 0.0001$; interaction: $F_{18, 126} = 0.60$, $p = 0.89$; Fig. 2). The comparison of the different series revealed a significant dose effect. Irrespective of the developmental stage considered, Tukey's HSD test detailed three groups ($M_C + F_C > M_T + F_C > M_C + F_T = M_T + F_T$). This result reveals a greater sensitivity of females to Azadirachtin.

In addition, for all stages evaluated, a dose-dependent effect was noted and a more significant reduction was recorded at the LD_{50} than at LD_{25} (supplementary Fig. 1). For homogenization of the results along the core of the paper, the values of treated series with LD_{25} are not presented in figure 2.

3.3 Cyst parameters

The effect of Azadirachtin treatment was tested on male fecundity. Azadirachtin was applied topically at LD_{50} (0.63 µg) on male *D. melanogaster* on the day of emergence. The number of cysts per testis was counted 48 hours after emergence. Control adults displayed 28.47 ± 2.16 cysts per testis. This number decreased significantly after topical application of Azadirachtin and reached 20 ± 1.69 ($t_{58} = 3.08$; $p = 0.003$) (Fig. 3A). In addition, the percentage of spermatocyte nuclei in abnormal position significant increased in LD_{50} males ($\chi^2 = 5.30$, 1; $z = 2.30$, $p = 0.021$) (Fig. 3B).

3.4 Ovarian parameters

The effect of Azadirachtin treatment was tested on female fecundity. Azadirachtin was applied topically at LD_{50} (0.63 µg) on female *D. melanogaster* on the day of emergence. The number of oocytes was counted 48 hours after emergence. We found a significant decrease in the number of oocytes in the treated series (13.90 ± 0.55) compared to controls (16.57 ± 0.53; $t_{58} = 3.43$; $p = 0.0011$, Fig. 4A). Furthermore, the volume of the basal oocyte showed a reduction in treated females (0.0048 ± 0.0003 mm^3) compared to controls (0.0071 ± 0.0005 mm^3) ($t_{58} = 3.87$; $p = 0.0003$, Fig. 4B).

4. DISCUSSION

In this study, we investigated Azadirachtin effects on adult toxicity and reproduction in *D. melanogaster*. Azadirachtin, applied topically on newly emerged adults, induced comparable mortality when evaluated at different times (24 – 96 hours) after treatment. This result is in agreement with those of Andreazza *et al*.[45] on two other Drosophilidae, *Drosophila suzukii* (Matsumara) and *Zaprionus indianus* (Gupta). Azadirachtin acts with a dose-response relationship and the LD_{50} we obtained (0.63 µg or 630 ppm, evaluated 24 h after treatment) is close to that found by Bezzar-Bendjazia *et al*.,[38] despite the fact that these authors used topical application at the last larval stage (0.67 µg). Azadirachtin seems less toxic (1.17 µg) when applied on newly formed pupae of *D. melanogaster*.[39] Neem-oil, another Azadirachtin formulation applied topically at the same stage, induces similar toxicity in *Drosophila*[39] in spite of two antifeedants (nimbine and salanine) in its composition. This can be explained by the mode of application; indeed, oral administration could have provided different results. The most likely explanation for this effect is a lower susceptibility due to the slow metabolism at this stage of development. Nevertheless, the pupal stage, a critical phase for adult formation, remains very responsive to Azadirachtin,[39] which inherently induces a drastic remodeling of most tissues and organs.[46]

Azadirachtin presents a stronger toxicity in *D. suzukii* and *Z. indianus* adults comparatively to *D. melanogaster*.[45] Indeed, a concentration of only 12 ppm causes 40% mortality in those species. Similar observations were reported by De Andrade-Coelho *et al.*[47] in female adults of another Diptera *Lutzomyia longipalpis* (Lutz and Neiva). Previously, Azadirachtin was reported to impair, with variable effects, the survival of different insect species.[11-14,48] Strong variations in insects' susceptibility to Azadirachtin were noted depending on insect order, species, formulation, or the method of application.[12,26,28,49-51] Sensitivity of insects to

insecticides varies also with the regulation of receptors,[52] penetration rate through the cuticle, absorption by insects, transport in tissues of the body and metabolism.[53]

Several mechanisms may be at play in causing acute Azadirachtin toxicity. Azadirachtin effects on adult mortality may be linked to the cytotoxicity and induction of apoptosis causing general disruption in the organs of *D. melanogaster*,[54,55] and in others species, like *Spodoptera frugiperda*.[9,56] As supposed by Lai *et al*.,[18] Shao *et al*.[54] revealed that Azadirachtin induced apoptotic cell death *via* insulin receptors and its signaling pathways.

Mortality can increase in *D. melanogaster* if the nutritional balance is impacted[57] and this parameter might be affected by Azadirachtin. Indeed, in *Drosophila*, rhythmic activity of feeding muscles is controlled by specific motor neurons[58] and cytotoxicity can be explained by a blockage of voltage-gated calcium channels and their impact on cholinergic synapses.[34] Azadirachtin could also induce morphological and physiological damages on the salivary glands, as was noted by Remedio *et al*.[36] in the mite (*Rhipicephalus sanguineus*, Latreille). In addition, the metabolic fight against the insecticide's toxicity[13,39] may involve a high energy requirement.[59] These processes affecting the flies' nutritional state, immediately following metamorphosis, could be potentially fatal and may contribute to the mortality observed in this study.

Quantitative and qualitative variations in nutrition significantly influence reproductive physiology and behavior as well as overall reproductive success.[60] In *Drosophila*, disruptions in energy homeostasis severely affect reproduction[61-63] and a correct lipid composition is crucial for spermatogenesis.[64] Effects of Azadirachtin on fecundity and oviposition have been reported in *D. melanogaster*,[38,39] other Diptera[31,32] and several insects orders.[9,12,28,51] Observations made in the current study indicate possible causes for these reprotoxic effects. Indeed, the fecundity and fertility of *D. melanogaster* that survived Azadirachtin treatment was adversely affected, resulting in a decreased progeny, and these depressive effects were

positively correlated with a reduction of cyst and oocyte numbers, an increase in spermatocyte nucleus anomalies and a decrease in the size of basal oocytes, all of which can explain the reduced progeny of treated adults. Females of *Drosophila* seem more sensitive than males in spite of a similar toxicity; this may be explained by a distinct physiology between the sexes. It is known that Azadirachtin causes significant effects on the reproductive system of both male and female insects by causing alterations and morphological changes in oocytes and spermatocytes.[9,35] In addition, the reduction of the egg layer can be explained by the neurotoxic action of Azadirachtin *via* the role of follicular adrenergic signaling in ovulation, demonstrated recently in *D. melanogaster*.[65]

The diminution of the size of basal oocytes in females, in agreement with several authors,[9,12,28,48] may be linked to a vitellogenesis impairment already noted in *D. melanogaster* after Azadirachtin treatment.[39] Azadirachtin alters or prevents the formation of a new actin cytoskeleton, resulting in disruption of cell division and blocked transport,[9] which may affect the dumping of cytoplasmic contents of nurse cells to the oocyte.[66] Azadirachtin can interfere with vitellogenin synthesis and/or its uptake, affecting oocyte development *via* juvenile hormone (JH). For the normal progress of oogenesis, vitellogenesis and spermatogenesis, a proper balance between JH and 20-hydroxyecdysone (20E) is of paramount importance in *D. melanogaster.* [66,67]

In recent literature, ecdysone or JH signaling pathways seem implicated in the regulation of microvilli formation in follicular epithelial cells[68] or in intestine changes (enterocyte remodeling) to yield a larger organ to support increased lipid metabolism.[69] Consequently, the impairment of flies' reproductive capacity could be explained by an antagonist action of Azadirachtin on these two essential hormones. The reproductive process is linked to nutritional signals controlled by the insulin signaling pathways, which are situated within a complex physiological regulatory network that also encompasses the lipophilic hormones, 20-

hydroxyecdysone and JH.[70,71] Furthermore, direct action of Azadirachtin on the nervous system[34] can also interfere indirectly on different endocrinological processes in insects.[72]

In conclusion, our study shows strong toxic effects of Azadirachtin, from 24 hours after treatment, on the non-target species *D. melanogaster*; this species, however, is less sensitive than the targeted species.[11,45,47] This study also highlights a more drastic impact of Azadirachtin on progeny output in females compared to males. Further investigations are needed to achieve a better understanding of these effects.

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Table 1. Toxicity of Azadirachtin tested by topical application, at different doses, on the day of *Drosophila melanogaster* adult emergence: lethal doses and their fiducial limits.

	Doses (µg)	**Fiducial limits**
LD 90	**4.85**	**[1.47– 15.98]**
LD 50	**0.63**	**[0.44 – 0.91]**
LD 25	**0.23**	**[0.13 – 0.38]**
Hill slope	**1.08 [0.54–1.61]**	
R square	**0.96**	

Table 2. Toxicity of Azadirachtin tested by topical application, at different doses, on the day of *Drosophila melanogaster* adult emergence: corrected mortality (%) from 24 to 96 hours (mean ± SD; n = 3 replicates of 300 insects). Means followed by the same uppercase letter do not differ significantly according to Tukey's HSD test at level $p \leq 0.05$.

Times (h) / Doses (µg)	24	48	72	96
0.1	13.92 ± 1.43 A	15.96 ± 2.19 A	14.72 ± 2.24 A	14.65 ± 0.97 A
0.2	26.16 ± 3.58 B	23.67 ± 1.13 B	28.12 ± 1.22 B	27.77 ± 5.84 B
0.4	34.84 ± 0.44 C	33.85 ± 4.61 C	35.02 ± 5.92 C	34.89 ± 4.57 C
0.6	43.16 ± 0.74 D	42.46 ± 2.49 D	43.42 ± 2.47 D	43.87 ± 6.90 D
1.2	71.80 ± 3.11 E	69.88 ± 1.13 E	70.45 ± 6.20 E	69.88 ± 1.16 E

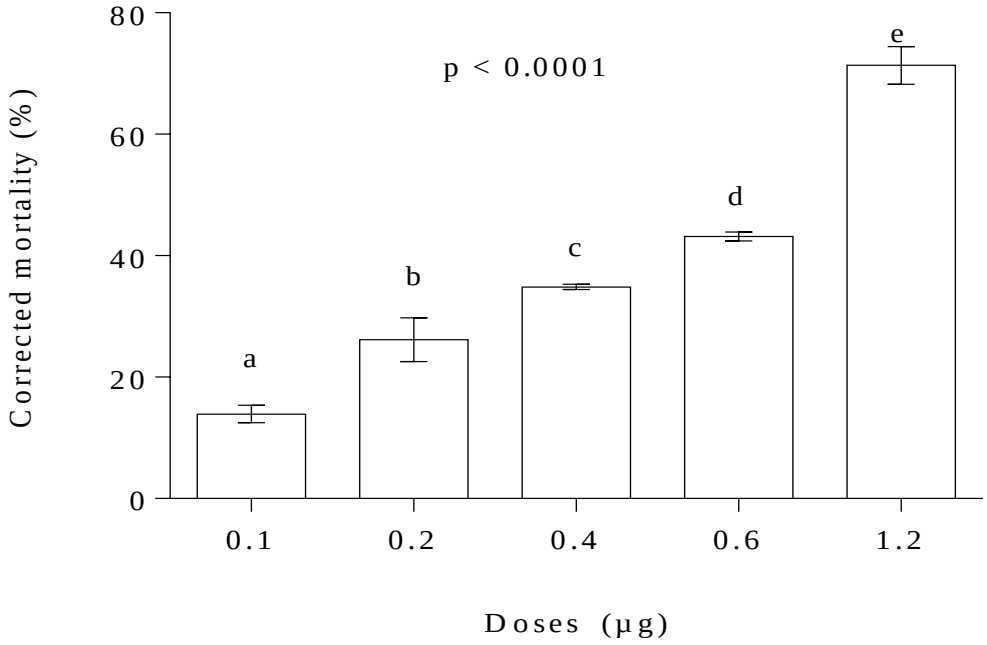


Figure 1. Toxicity of Azadirachtin tested by topical application, at different doses, on the day of *Drosophila melanogaster* adult emergence: corrected mortality (%) at 24 hours (mean ± SD; n = 3 replicates of 300 insects). Control mortality: 5.48 ± 0.53 %. Different letters indicate a significant difference between control and treated series according to Tukey's HSD test.

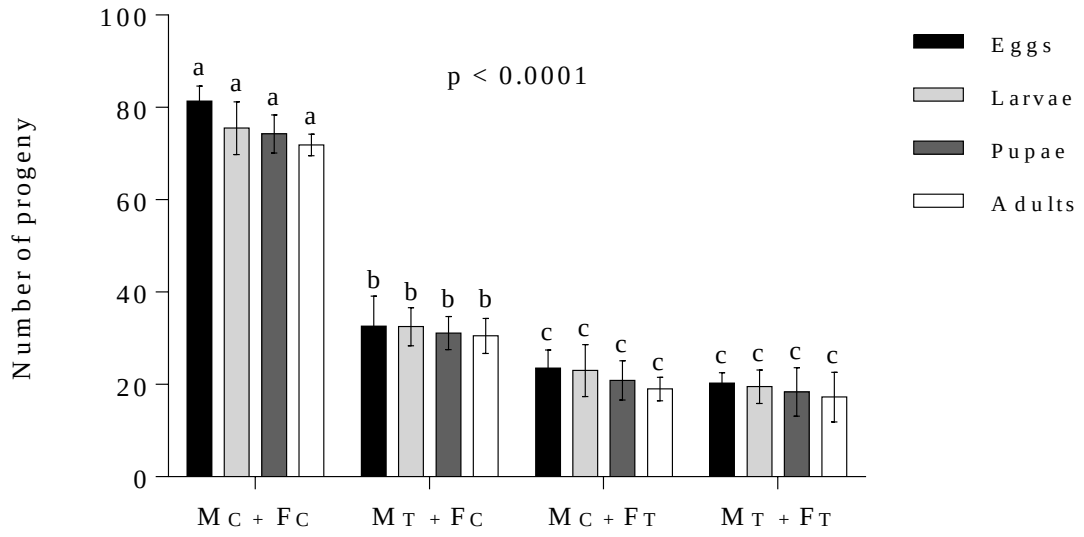


Figure 2. Effect of Azadirachtin tested by topical application (0.63 µg), on the day of *Drosophila melanogaster* adult emergence, on the number of progeny (eggs, larvae L3, pupae, adults; M_C: male control; F_C: female control; M_T: male treated; F_T: female treated) (mean ± SD; n = 8 by couple). Different letters indicate a significant difference between the control and treated series of the same stage of development at level $p \leq 0.05$.

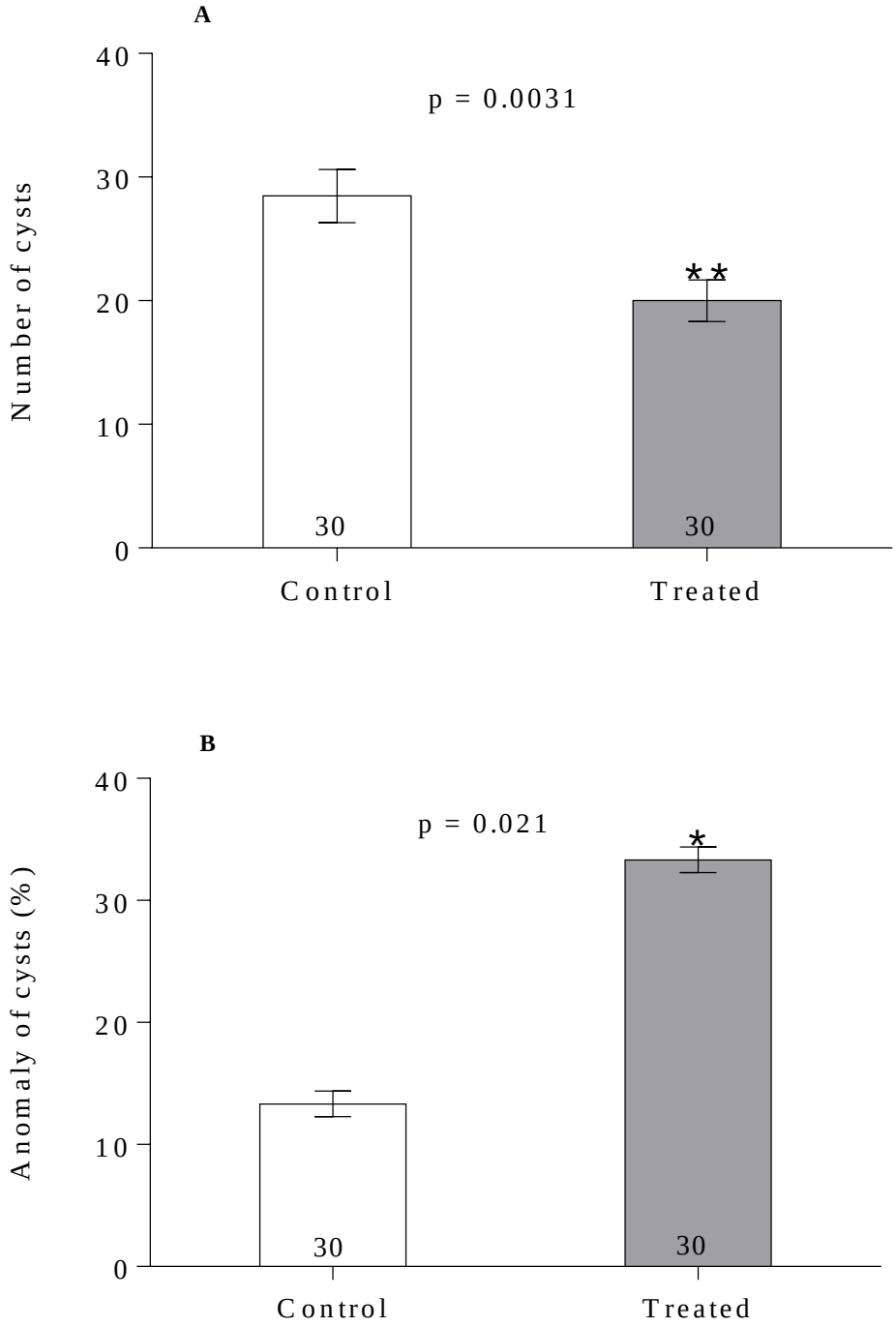
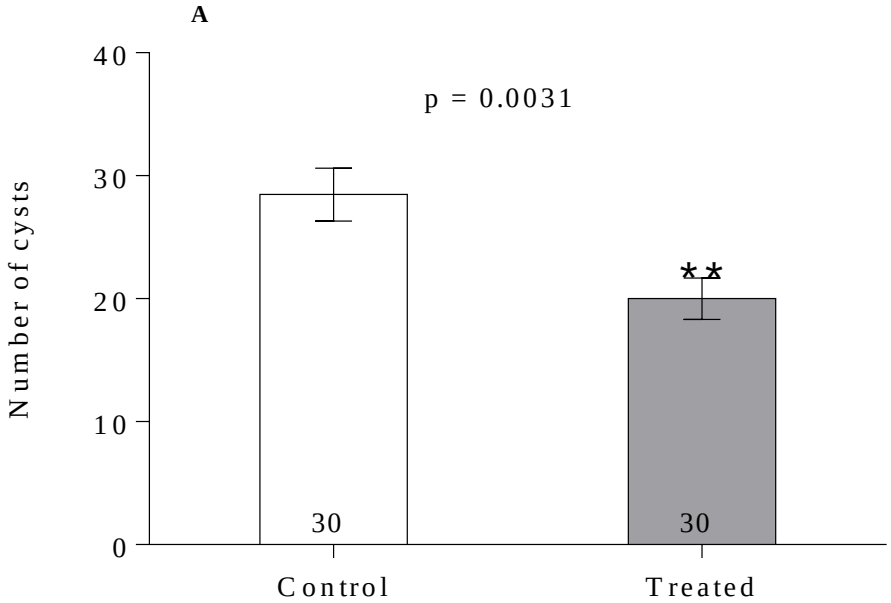


Figure 3. Effects of Azadirachtin applied by topical application (0.63 µg) on day of *Drosophila melanogaster* adult emergence on male fecundity parameters (mean ± SD). **A:** Number of cysts per testis 48h after emergence. **B:** Percentage of spermatocyte nuclei in abnormal position. Numbers within each bar indicate the number of repetitions;* above bars indicate significant difference at $p \leq 0.05$;** above bars indicate significant differences at $p \leq 0.01$.



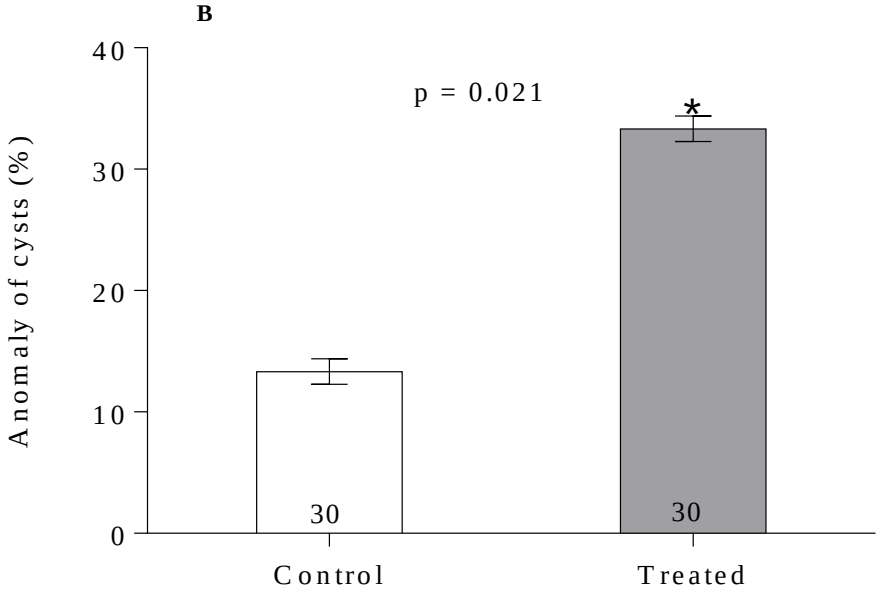
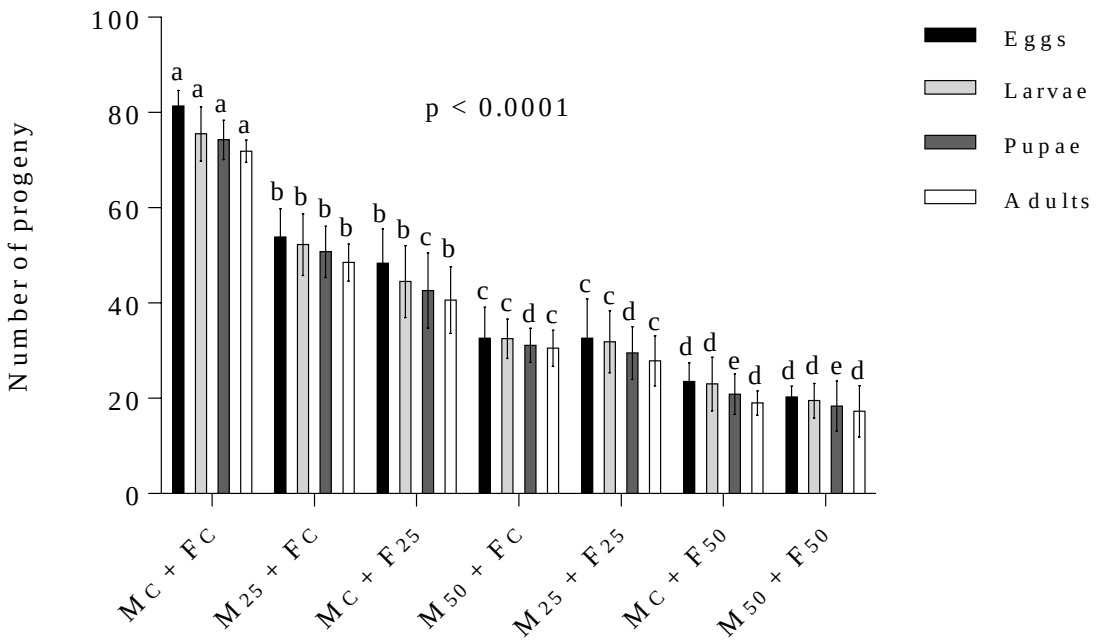


Figure 4. Effects of Azadirachtin applied by topical application (0.63 µg) on day of *Drosophila melanogaster* adult emergence on female fecundity parameters (mean ± SD). **A:** Number of oocytes per ovary 48h after emergence. **B**: Volume of the basal oocyte 48h after emergence. Numbers within bar indicate the number of repetitions;** above bars indicate significant differences $p \leq 0.01$. *** Above bars indicate significant differences $p \leq 0.001$.

Supplementary figure 1



Effect of Azadirachtin tested by topical application (LD_{25}: 0.23 µg and LD_{50}: 0.63 µg), on the day of *Drosophila melanogaster* adult emergence, on the number of progeny (eggs, larvae L3, pupae, adults; M_C: male control; F_C: female control; M_{25} and M_{50}: male treated at LD_{25} and LD_{50}; F_{25} and F_{50}: female treated at LD_{25} and LD_{50}) (mean ± SD; n = 8 by couple). Different letters indicate a significant difference between control and treated series at the same stage of development at level $p \leq 0.05$.